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<b>(21) International Application Number:</b> PCT/GB96/02095 <b>(22) International Filing Date:</b> 29 August 1996 (29.08.96) <b>(30) Priority Data:</b> 9517585.7 29 August 1995 (29.08.95) GB <b>(71) Applicant (for all designated States except US):</b> ISIS INNOVATIONS LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> COOKSON, William, Osmond, Charles [GB/GB]; 67 Hilltop Road, Oxford OX4 1PD (GB). HILL, Michael, Richard [GB/GB]; 27 Ridgeway Road, Risinghurst, Oxford OX3 8DS (GB). <b>(74) Agents:</b> O'BRIEN, Caroline, Jane et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ATOPY: DIAGNOSIS AND THERAPY  <b>(57) Abstract</b>  The invention relates to a method for diagnosing an individual as having atopy or a predisposition thereto. The method involves demonstrating in the individual the presence or absence of either: (i) an unusual variant form of the gene which codes for the beta sub-unit of the high affinity receptor for IgE (Fc $\epsilon$ R1 $\beta$ ), the variant form comprising a variation in exon (7) which encodes a glycine at amino acid residue 237 instead of glutamic acid which appears at residue 237 in individuals without atopy; or (ii) an unusual polymorphic form of the amino acid sequence for Fc $\epsilon$ R1 $\beta$ , the polymorphic form comprising glycine at amino acid residue 237 instead of glutamic acid which appears at residue 237 in individuals without atopy. Materials and other methods relating to the above diagnostic method are also described.		

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## ATOPY: DIAGNOSIS AND THERAPY

5 The present invention relates to the diagnosis and therapy of atopy and to materials and methods relating thereto.

Atopy is in essence an excessive or misplaced immune response mediated by IgE antibodies in response to common environmental allergens. Common allergens sensitizing atopic individuals are grass and tree pollens, house dust, house dust  
10 mites (HDM), bacteria, fungi, feathers, hair, eggs, milk and chocolate. Atopy underlies hayfever, asthma, eczema, urticaria and certain gastro-intestinal disorders.

Asthma is an atopic condition of particular interest as it is becoming more  
15 prevalent. It now affects one child in seven in the United Kingdom (Strachan D.P., et al., Arch. Dis. Childhood 70, 174-178, 1994). Allergies/atopy underlies 95% of childhood asthma cases. Asthma may be identified by recurrent wheeze and intermittent airflow limitation. An asthmatic tendency can be quantified by the measurement of bronchial hyper-responsiveness (BHR) in which an  
20 individual's dose-response curve to a broncho-constrictor such as histamine or methacholine is constructed. The curve is commonly summarised by the dose which results in a 20% fall in airflow (PD20) or the slope of the curve between the initial airflow measurement and the last dose given (slope).

25 In an atopic immune response, IgE is produced by B-cells in response to allergen stimulation. These antibodies coat mast cells by binding to the high affinity receptor for IgE (FcεRI). When a multivalent allergen binds to an IgE coated mast cell, the cross-linking of adjacent IgEs by allergen initiates a series of cellular events leading to the destabilization of the cell membrane and release of  
30 inflammatory mediators. This results in mucosal inflammation, wheezing, coughing, sneezing and nasal blockage.

Atopy can be diagnosed by (i) a positive skin prick test in response to a common allergen; (ii) detecting the presence of specific serum IgE for allergen; or (iii) by detecting elevation of total serum IgE. Using these and other assays, a genetic linkage between generalised atopic IgE responses and chromosome 11q has been observed.

Previous studies have found linkage of atopy and bronchial hyper-responsiveness to markers on chromosome 11q13 (eg Cookson, W.O.C.M., et al., 1989 Lancet i pages 1292-1295.) and the beta-chain of the high affinity receptor for IgE (Fc $\epsilon$ RI $\beta$ ) has been identified as a candidate gene for this linkage (Sandford, A.J., et al., 1993 Lancet 341, pages 332-334).

WO95/05481 provides further information on atopy and its diagnosis and treatment. In particular, it discloses variants of the gene encoding Fc $\epsilon$ RI $\beta$  which are associated with atopy and it teaches a method for diagnosing atopy which is based upon the demonstration of the presence or absence of one of two variants in a specific portion of the DNA sequence of the gene encoding Fc $\epsilon$ RI $\beta$ . The specific DNA sequence is located near the commencement of exon 6 of the Fc $\epsilon$ RI $\beta$  gene on chromosome 11q and two variants are described. The first variant (designated I181L) encodes a polymorphism in which there is a change of isoleucine to leucine at amino acid residue 181. The second variant (designated I181L/V183L) involves a double mutation that includes the change at residue 181 and the additional change of valine to leucine at residue 183. The amino acid residues 181 and 183 are positioned in the fourth transmembrane domain of Fc $\epsilon$ RI $\beta$ . The variants and polymorphisms are fully described in WO95/05481.

The polymorphisms I181L and I181L/V183L and associated polynucleotide variants have been described in British and Australian populations and are, in selected subjects, associated with atopy and atopic asthma (Shirakawa, T., et al. 1994 Nature Genetics 7, 125-9; and Hill, M. R., et al., 1995 Br. Med. J., 311, 776-779). However the two polymorphisms/variants I181L and I181L/V183L have in practice proved problematical to assay, there being a high false-negative

rate for PCR-based tests.

Unexpectedly, the present applicants have found a further variant associated with atopy and atopic asthma which is in the coding sequence for the C-terminal cytoplasmic tail of FcεRIβ (the first quarter of the C-terminal tail is encoded in exon 6 and the remainder in exon 7). This new variant (designated E237G) is located within exon 7 of the FcεRIβ gene. The normal sequence of the FcεRIβ gene has been published in Kuster, H., et al. 1992, J. Biol. Chem., 267, 12782-12787, "The gene and cDNA for the human high affinity immunoglobulin E receptor β chain and expression of the complete human receptor". The sequence can also be found in the Genbank/Embl Databases under Accession No. M89796.

The newly discovered variation in the coding sequence for the C-terminal cytoplasmic tail of FcεRIβ results in a change of glutamic acid to glycine at amino acid residue 237. Sequencing of exon 7 in atopic individuals confirms a nucleotide change from adenine to guanine at nucleotide residue 6843 of the FcεRIβ gene (Kuster et al., 1992 supra.). For completeness it should be noted that nucleotide residue 6843 of the 1992 Kuster et al sequence, corresponds to nucleotide residue number 7297 in the Genbank/Embl Database sequence.

To date the applicants have investigated 1004 Australian Caucasian individuals in 230 nuclear families for the presence of the E237G polymorphism. 26 males and 26 females were found to have the polymorphism giving a population prevalence of about 5.3%. The E237G polymorphism was significantly associated with increases in (i) skin test responses to house dust mite ( $p=0.02$  by Mann-Whitney U test) and grass pollen ( $p=0.0008$ ); (ii) radioallergosorbent test specific IgE titres (RAST) to HDM ( $p=0.001$ ) and grass pollen ( $p=0.04$ ); and (iii) BHR to metacholine ( $p=0.0006$ ). The relative risk of children carrying the E237G polymorphism having asthma was 2.4 compared to children without the variant (95% CI 1.29-4.32;  $p=0.004$ ). Unlike the I181L and I181L/V183L polymorphisms which are maternally linked, the E237G polymorphism is independent of the parental origin of the allele.

In a similar study of individuals in Japan, the E237G polymorphism has also been found to be associated with atopic asthma, there being a particularly high association with childhood asthma (Shirakawa, T. et al., 1996 Human Molecular Genetics, Vol.5, No. 8 pages 1129 to 1130.).

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The high-affinity receptor for IgE, FcεRI, is a tetrameric complex formed by an α chain which can bind IgE, a β chain and a homodimer of γ chains. It belongs to a family of receptors that contain homologous activation motifs that appear capable of autonomously triggering cell activation via protein-tyrosine phosphorylation.

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Two forms of the activation motif appear in FcεRI: one in the β chain and one in the γ chain. They are believed to operate synergistically. Upon triggering of FcεRI, the protein-tyrosine kinase Lyn, already associated with the β chain becomes activated and phosphorylates the β and γ chains. Phosphorylation of the

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γ chain induces the association of protein-tyrosine kinase Syk with the γ chain and the subsequent activation of Syk that then activates phospholipase (C<sup>v</sup> 1 leading to calcium mobilization). The β chain cannot initiate the full signalling capacity of FcεRI. However the importance and unique role of the β chain is demonstrated when the cytoplasmic C-terminal tail is removed with the consequence that

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signalling is abolished even when the γ dimer is present. This indicates a role for the β chain in controlling receptor phosphorylation. Amino acid residue 237 (the site of the polymorphism) is located adjacent to the immunoreceptor tyrosine activation motif (residues 212-228) reported in the cytoplasmic C-terminal tail of FcεRIβ. The polymorphism E237G introduces a significant hydrophobicity change within the C-terminus of FcεRIβ which may effect the intracellular signalling

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capacity of FcεRIβ. The substitution of the polar negatively charged glutamic acid at residue 237 with the smaller polar, but uncharged, glycine results in the loss of the hydrophilic nature of this region when examined by the Chou-Fassman hydrophobicity prediction program (GCG Molecular Biology Package, Wisconsin).

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Thus the present applicants have unexpectedly established that there is a previously unrecognised variant/polymorphism (in the nucleotide/amino acid sequences respectively) in the cytoplasmic C-terminal tail of FcεRIβ which comprises

sequence changes associated with a change of glutamic acid to glycine at amino acid residue 237 and which is associated with atopy and atopic asthma. These new and unexpected teachings make possible new diagnostic methods.

5 Thus the present invention provides a method for diagnosing an individual as being atopic or asthmatic, or of having a predisposition to atopy or asthma which comprises demonstrating in the individual the presence or absence of an unusual variant form of the gene which codes for  $Fc\epsilon RI\beta$ , the variant form encoding a polymorphism comprising glycine at residue 237 instead of glutamic acid.

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As an alternative to analysing the gene for a variant, the amino acid sequence may be analysed for the equivalent polymorphism.

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Thus the present invention also provides a method for diagnosing an individual as being atopic or asthmatic or of having a predisposition to atopy or asthma which comprises demonstrating in the individual the presence or absence of an unusual polymorphic form of the amino acid sequence for  $Fc\epsilon RI\beta$ , the polymorphic form comprising a glycine at residue 237 instead of glutamic acid.

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The presence or absence of an unusual polymorphic form of the amino acid sequence for  $Fc\epsilon RI\beta$  may be determined by use of a substance which comprises an antibody binding domain which specifically/selectively binds to the unusual polymorphic form which comprises glycine at residue 237 instead of glutamic acid, but not to the normal form of  $Fc\epsilon RI\beta$  which occurs in individuals without symptoms of atopy/asthma. The substance may comprise an antibody, which may be polyclonal or monoclonal.

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Where analysis is made at the genetic level, this may be done by employing a technique using one or more nucleic acid sequences which are able to hybridise to the unusual variant form of the gene under suitable stringency conditions.

Typically, one or more of such sequences may be used as primers in a detection system comprising a polynucleotide amplification technique such as PCR, ARMS.

The amplification primers may be specific to the unusual variant, but not to wild-type. In which case amplification will only occur if the unusual variant is present in the test sample. Alternatively, the amplification primers may be non-specific. In which case they are used to amplify a sequence region of the variant gene  
5 which comprises the nucleotide change(s) which effect the amino acid change at residue 237. Thus an unusual variant such as E237G may be detected by specific PCR using the established techniques of direct sequencing, SSCP and ARMS (Sanger F., et al., 1977 PNAS USA 74: 5463-5467; Orita M., et al., 1989 Genomics 5: 874-879; Newton C.R., et al., 1989 Nucl. Acids Res., 17: 2503-  
10 2516).

PCR, ARMS etc may be followed by direct sequencing and sequence analysis, analysis of single stranded conformational polymorphism (SSCP) and/or size analysis of the amplification products where the amplification reaction is designed  
15 to amplify a nucleotide sequence the size of which is characteristic of the presence of the unusual variant comprising the nucleotide changes and/or by probing the amplification products with a sequence-specific nucleic acid probe capable of annealing to a portion of the amplified gene which is specific to the unusual variant and not to wild-type. Any suitable method for the confirmation of the  
20 existence of a particular nucleotide sequence may be employed and the suggestions given above are for the purposes of illustration only.

DNA or RNA-based amplification methods may be employed. Thus the amplification may be carried out based on mRNA or cDNA made from mRNA.  
25

If primers used are specific to the variant, there will only be successful amplification by eg PCR, ARMS if the variant is present in the test sample. Alternatively the primers used could be non-specific to the variant, the amplification serves only to increase sample material for analysis by a different  
30 method such as direct sequencing and SSCP as mentioned above. Suitable nucleic acid sequences for use as specific probes, or specific or non-specific primers in a detection assay for E237G variant/polymorphism as discussed.



herein can be ascertained from knowledge of the known DNA sequence (Kuster et al., 1992 supra.) and the disclosure of the existence of the variant/polymorphism as provided herein and come within the scope of the present invention.

5 The teaching of the existence of the E237G variant/polymorphism enables one skilled in the art to easily design and make in accordance with known techniques eg probes and primer pairs based on the publicly available sequence information for FcεRIβ. Similarly a skilled person would be able to ascertain from the publicly available sequence information for FcεRIβ, peptides and polypeptides  
10 which may be made and employed in accordance with known techniques, to produce substances comprising an antibody binding domain which specifically/selectively bind to the E237G polymorphic form and fail to bind substantially to the normal form of FcεRIβ which occurs in individuals without symptoms of atopy/asthma.

15 The patient sampling and assay system (whether assaying polynucleotide or amino acid sequences) may be in accordance with standard techniques in the art. However such assay systems will be newly utilising complementary nucleic acid sequences as probes or primers, or specific antibodies identified by the applicants  
20 by virtue of the disclosure of the E237G variant/polymorphism made herein as being useful for the diagnosis of atopy or asthma or a predisposition there towards.

Thus the present invention also provides oligonucleotide hybridisation probes  
25 which comprise a sequence of nucleotides with sufficient complementarity and length to enable specific hybridization under suitably high stringency conditions. to a portion of test sample genetic material deriving from an individual patient which comprises the variation in the sequence which gives rise to an unusual E237G form of the gene for the beta sub-unit as discussed above; the probe itself may  
30 hybridise to the unusual sequence of nucleotides which can give rise to the variant gene. The oligonucleotide hybridisation probe may not show significant hybridisation to an equivalent portion of test sample genetic material derived from

an individual without symptoms of atopy/asthma.

A probe should comprise sufficient nucleotides for efficient and specific annealing in order that the diagnostic analysis be sufficiently accurate. Typically a probe  
5 may comprise 17 to 20 nucleotides, but of course the skilled person could prepare probes of other lengths and test them in accordance with known techniques and the disclosures herein for functionality in a given set of suitably high stringency hybridisation conditions. In order to aid detection of specific hybridisation, the probe may be labelled in accordance with techniques well-known in the art.

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In the alternative, there are provided nucleic acid sequences with sufficient complementarity and length to enable specific hybridization under suitably high stringency conditions to a portion of test sample genetic material which comprises the variation in the sequence which gives rise to the E237G form of the gene for  
15 the beta sub-unit as discussed above, for use as primers in a reaction to amplify the gene. Typically there may be provided a pair of such primers. The amplification reaction may comprise one stage of a diagnostic assay. The hybridization of such primers under suitably high stringency conditions may be to a portion of nucleotide sequence lying in the vicinity of, but upstream and/or  
20 downstream of the E237G variant nucleotides which give rise to the unusual form of  $Fc\epsilon RI\beta$ . The primers may be non-specific for the E237 variant nucleotides or a primer may be specific for the E237 variant nucleotides. The methods as herebefore described may utilise nucleic acid sequences, probes and primers both as generally described above, or of the specific examples (or functional equivalents  
25 thereof) given in the detailed description which follows.

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Reference above is made to high-stringency conditions for hybridization. Hybridization is the process whereby two single-stranded polynucleotides anneal to each other to form a double-stranded molecule. The annealment is effected by  
hydrogen bonding between the complementary bases in the two strands. However  
under certain reaction conditions (non-stringent conditions) any pair of single-stranded polynucleotides will anneal to each other to form a double-stranded

molecule (non-specific hybridization). Under different reaction conditions (high stringency conditions) this non-specific hybridization is reduced or minimised so that hybridization occurs, only between a pair of single-stranded polynucleotides with substantial (if not complete) complementarity to each other. The more stringent the conditions are, the greater complementarity is needed for hybridization. As stringency conditions are decreased, so does the requirement for complementarity.

Thus the skilled person will in accordance with standard techniques identify a set of reaction conditions sufficiently stringent for their particular purpose. Generally of course the aim is to identify by use of one single-stranded polynucleotide sequence as a probe or primer another single-stranded complementary sequence in a test sample which is either exactly complementary or substantially complementary to the probe or primer sequence. The reaction conditions (the high stringency condition) at which the hybridization is adequately specific will also be determined by the length of the single-stranded polynucleotide sequence used as a primer or probe. Where the primer/probe sequence is very short, greater care may be needed in order to avoid non-specific binding and the reaction conditions therefore may need to be highly stringent. The reaction conditions however may need to be less stringent where the primer/probe sequence is long (and therefore likely to be very specific).

It can be seen from the above that 'high-stringency conditions' are variable according to the nature of the primer/probe and whether or not the skilled person is desirous of identifying only exactly complementary sequences or of sequences with incomplete complementarity. The skilled person in accordance with standard techniques is able to identify for themselves the high stringency conditions appropriate to their experimental or diagnostic aim.

Analysis may also be made of the amino acid sequence. This may be done by sequencing studies, or by use of a probe comprising an antibody binding domain which is specific for the E237G polymorphism. The probes may comprise

antibodies (monoclonal or polyclonal) which may be raised against the unusual E237G polymorphism eg by use of peptides/polypeptides ascertained and made in knowledge of the available sequence information for FcεRIβ as mentioned above. The probes may be labelled in accordance with standard methodologies. In vitro or in vivo diagnosis may be possible. Thus the present invention also provides probes comprising an antibody binding domain which are specific for the E237G polymorphism. Such 'antibody binding domain' probes may not bind to the normal form of FcεRIβ present in individuals without symptoms of atopy/asthma. The probes may be antibodies. They may be polyclonal or monoclonal.

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As mentioned above, given knowledge of the amino acid sequence for FcεRIβ and the unusual E237G polymorphic forms associated with atopy or asthma described herein, standard synthesis techniques may be used to manufacture short peptides or longer peptides characteristic of the E237G polymorphism and not of the normal 'wild-type' form in individuals without atopy, for use as immunogens to raise antibodies. In the alternative, the E237G polymorphism associated with atopy or asthma and described herein may be isolated and purified for use as an immunogen to raise antisera. The present invention also therefore provides polypeptides (synthetic or isolated) and immunologically cross-reactive derivatives and fragments eg short peptide sequences which are characteristic of the E237G polymorphism as specifically described herein.

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The present invention also provides diagnostic kits and reagents for the detection of atopy or asthma or a predisposition there towards, comprising any of nucleic acid sequences, probes, primers, antibodies, polypeptides or peptides as described above. Also provided is the use of any of nucleic acid sequences, probes, primers, antibodies, polypeptides or peptides as described above in methods as described, or in the manufacture of diagnostic kits and reagents for the detection of atopy or asthma or a predisposition there towards.

In terms of treatment possibilities the disclosure that the E237G polymorphic form of the cytoplasmic C-terminal tail of FcεRIβ is associated with atopy or asthma,

enables assays to screen for compounds which down-regulate the unusual polymorphic form. The sequence information provided herein will also enable the development of antisense RNA treatment strategies to block the E237G polymorphism of the cytoplasmic C-terminal tail of  $Fc\epsilon RI\beta$ . Antibody based therapies focussing on the E237G polymorphism may also be possible. Use may be made of an antibody, possibly humanised in accordance with known techniques, or of a compound comprising just an antibody domain which is specific for the E237G polymorphism for treatment of an individual with atopy or asthma or a predisposition there towards. In particular, the effect of the unusual polymorphic form of the cytoplasmic C-terminal tail of  $Fc\epsilon RI\beta$  on intracellular signalling may identify a novel target for therapeutic intervention.

Thus the present invention also extends to cover use of any of nucleic acid sequences, probes, primers, antibodies, polypeptides or peptides as described above in treatment strategies or in methods to identify compounds of prophylactic or therapeutic value. Invention also extends to any such prophylactic or therapeutic compounds identified by use of the information materials and methods disclosed herein.

In summary, detection of an unusual E237G polymorphic form of the beta sub-unit of  $Fc\epsilon RI\beta$  or detection of a coding sequence for said E237G polymorphism is a new and unexpected finding of diagnostic use in predicting/diagnosing atopy and specific atopic conditions such as asthma. If an E237G polymorphism/variant associated with atopy or asthma is detected in an individual preferably at the neonatal stage in accordance with the teachings of the present application, strategies for prevention of atopy or asthma by environmental manipulation to control contact with common allergens and/or vaccination against common allergens can then be devised for the individual concerned.

Furthermore, the E237G polymorphism may also define a subgroup of eg asthma sufferers with a particular clinical course, in which case recognition of the variant/polymorphism would be of value in defining prognosis and management of

asthma.

There now follows a more detailed description about testing for the E237G variant in FcεRIβ.

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Located in exon 7, E237G lies within the C-terminal cytoplasmic tail of FcεRIβ. The polymorphism is an amino acid change at residue 237 from glutamic acid to glycine. The polymorphism E237G is detected by eg specific PCR using the established techniques of direct sequencing, SSCP and ARMS.

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#### **(A) Direct sequencing and Single Stranded Conformational Polymorphism (SSCP)**

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SSCP is a technique in which DNA is amplified and then split into its two component chains and then moved down a gel by electrophoresis. The presence of a mutation on variant/polymorphism in one of the chains may mean that it forms a different shape (conformation) in the gel and so moves separately from the wild type thereby allowing detection.

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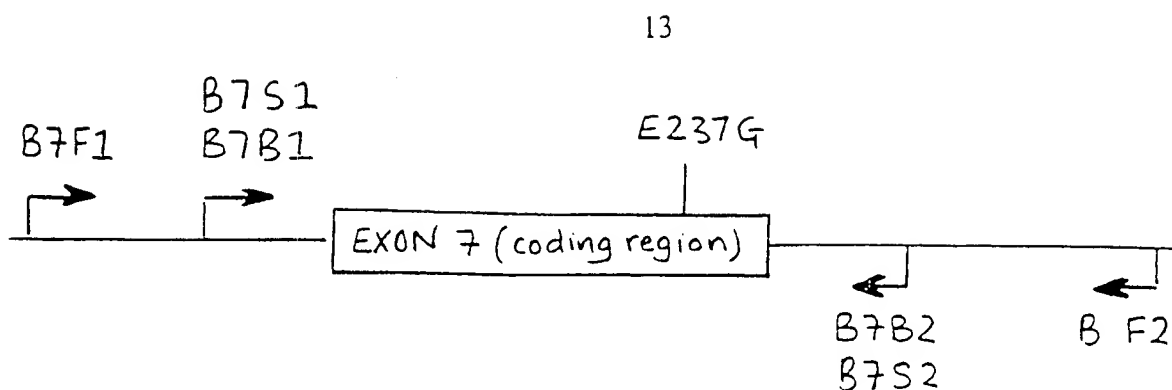
A sample of patient blood is taken and peripheral blood leucocytes obtained in accordance with standard techniques. DNA is extracted from the leucocytes by standard techniques (see eg Blin, N., et al., 1976 Nucleic Acids Research., Vol. 3, page 2303: "A general method for isolation of high molecular weight DNA from eukaryotes").

25

Direct sequencing and SSCP involves a two round PCR (two rounds is not essential, but it provides a cleaner product) with a common first round. For direct sequencing the second round incorporates a biotinylated primer.

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Schematically:

Primer pairsProduct size

	1st round	5'	3'	
10		B7F1:	CCA GCT AGT CTG GTT TGG TTT	515bp
		B7F2:	ATT AAG GTG GAC AGA AGC AGC	
	2nd round			
	5'-3' sequencing	B7S1:	GAT GAG GTA AGT CTC TTG AG	238 bp
15		*B7B2:	AAC CTT GGC CTT CTG GAT	
	3'-5' sequencing	*B7B1:	TGA GGT AAG TCT CTT GAG	238 bp
		B7S2:	CAA AAC CTT GGC CTT CTG G	
	SSCP	B7S1:	GAT GAG GTA AGT CTC TTG AG	240 bp
20		B7S2:	CAA AAC CTT GGC CTT CTG G	
	(*5' biotinylated primer)			

PCR reaction mixture

25	1st round	10μl per reaction volume:	Template is 50-100ng genomic DNA.
	2nd round	50μl per reaction volume:	Template is 1μl 1st round product diluted x100 in dH <sub>2</sub> O.
	Reaction mixture for 1st and 2nd round:		0.1μg each primer, 200μM dNTP, 50mM KCl, 10mM Tris-HCl (pH8.3), 1.5mM MgCl <sub>2</sub> and 0.2 units <i>Taq</i> polymerase (Boehringer Mannheim).
30			

PCR conditions

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**1st round**

94°C 5.0 minute 1 cycle  
 94°C 1.0 minute  
 59°C 1.0 minute 35 cycle  
 5 72°C 1.0 minute  
 72°C 10.0 minute 1 cycle

**2nd round**

94°C 5.0 minute 1 cycle  
 94°C 1.0 minute  
 50°C 0.5 minute 20 cycle  
 72°C 0.5 minute  
 72°C 10.0 minute 1 cycle

**(Ai) Direct sequencing****10 Preparation of single strand template DNA**

Single strand template is prepared from 40µl 2nd round PCR product using streptavidin coated magnetic beads (Dynabeads, Dynal UK Ltd) following manufacturer's instructions.

**15 Sequencing**

Performed using Sequenase Version 2.0 DNA Sequencing Kit (Amersham LIFE SCIENCE) and [ $\alpha$ -<sup>35</sup>S] dATP radionucleotide following suggested protocol.

Sequencing primer used is the non-biotinylated primer of the 2nd round PCR.

20 Sequencing in either direction detects E237G and it is sometimes useful to perform both as confirmation when heterozygote sequence appears faint.

**(Aii) SSCP**

25 Using Bio-Rad Protean II Cell apparatus (Bio-Rad Laboratories Ltd, UK), 4µl of second round PCR product was electrophoresed at 10 W per gel at 4°C for 22 hours in a 10 % (w/v) polyacrylamide (19: 1 acrylamide: bis)/10% (v/v) glycerol gel with 1x TBE buffer. DNA was visualized by silver staining using a Bio-Rad Silver Stain Kit (Bio-Rad Laboratories Ltd, UK) and then gels were vacuum-dried  
 30 onto Whatman 3M paper (Merck Ltd).

**(B) ARMS**



Primers

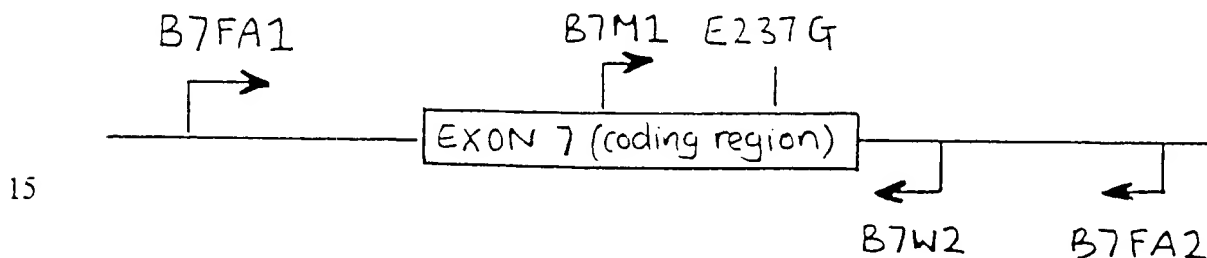
B7FA1: TGG CCA GCT AGT CTG GTT TGG TTT TCT GGA

5 B7FA2: GGA GCA TAT TAA GGT GGA CAG AAG CAG CAG

B7M1: ATT CAG CTA CTT ACA GTG AGT TGG AAG ACC CAG  
GCG G

B7W2: CAC GTG ATT CTT ATA AAT CAA TGG GAG GAG ACA ATT

10 Schematically:



20 PCR reaction mixture

50 $\mu$ l reaction volume: 0.1-0.2 $\mu$ g genomic DNA, 250ng of primers  
B7FA1, B7FA2, B7W2 and 125ng of primer  
B7M1, 200 $\mu$ M dNTP, 50mM KCl, 10mM  
Tris-HCl (pH8.3), 1.5mM MgCl<sub>2</sub> and 0.2  
25 units *Taq* polymerase (Boehringer Mannheim).

PCR conditions

30 A Hot start is recommended, *Taq* polymerase being added in 20% of the final  
volume of PCR reaction mixture after the first cycle which is held at 80°C.

94°C 5.0 minute 1 cycle (Hold at 80°C. add *Taq*)

16

94°C 1.0 minute

60°C 2.0 minute 35 cycle

72°C 2.0 minute

72°C 10.0 minute 1 cycle

5

Genotyping

Amplified product is visualized in an ethidium bromide stained 4% agarose gel (3.0g NuSieve GTG (Flowgen), 1.0g LMP agarose (GIBCO BRL), 50µg ethidium bromide per 100ml 0.5x TBE) run for two hours at 60V. In addition to the 446 bp control band, if present wild type sequence will result in a 280 bp band and E237G sequence in a 238 bp band.

For any additional general guidance on interpreting the detailed instruction given above, reference may be made to Sanger F. et al., 1977 supra., Orita M., et al., 1989 supra and Newton C.R., et al., 1989 supra., or to standard texts such as Short Protocols in Molecular Biology Second Edition A Compendium of Methods from Current Protocols in Molecular Biology edited by Frederick M. Ausubel et al. and Molecular Cloning, A Laboratory Manual eds Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989.

**CLAIMS:**

1. A method for diagnosing an individual as having atopy or a predisposition thereto, which comprises demonstrating in the individual the presence or absence of either: (i) an unusual variant form of the gene which codes for the beta sub-unit of the high affinity receptor for IgE (Fc $\epsilon$ RI $\beta$ ), the variant form comprising a variation in exon 7 which encodes a glycine at amino acid residue 237 instead of glutamic acid which appears at residue 237 in individuals without atopy; or (ii) an unusual polymorphic form of the amino acid sequence for Fc $\epsilon$ RI $\beta$ , the polymorphic form comprising glycine at amino acid residue 237 instead of glutamic acid which appears at residue 237 in individuals without atopy.
2. A method according to claim 1 which comprises the steps of:
  - obtaining a suitable tissue sample from the individual;
  - preparing from the tissue sample a nucleic acid sample; and
  - probing or analysing the nucleic acid sample for the presence or absence of said unusual variant form of the gene which encodes Fc $\epsilon$ RI $\beta$ .
3. A method according to claim 2 wherein prior to said probing or analysis, said gene which encodes the unusual variant form of the Fc $\epsilon$ RI $\beta$  gene is amplified.
4. A method according to claim 3 wherein amplification is by PCR.
5. A method according to claim 4 wherein the PCR amplification is carried out using a pair of oligonucleotide primers and a first member of the primer pair comprises a nucleotide sequence which hybridises under suitably stringent conditions to a substantially complementary nucleotide sequence which is proximal to, and 5' of, the codon for amino acid residue number 237 and the second member of the primer pair comprises a nucleotide sequence which hybridises under suitably stringent conditions to a substantially complementary nucleotide sequence which is proximal to, and 3' of, the codon for amino acid residue

number 237.

- 5 6. A method according to claim 3 or claim 4 wherein the amplification is carried out using a pair of oligonucleotide primers and a member of the primer pair comprises a nucleotide sequence which itself hybridises under suitably stringent conditions to the unusual sequence of nucleotides which give rise to said variant gene, such that amplification will only proceed when said variant gene is present in the sample.
- 10 7. A method according to any one of claims 2 to 6 which comprises probing the nucleic acid sample with an oligonucleotide hybridisation probe which under suitable conditions of high stringency only shows substantial hybridisation to said variant gene and not to the equivalent gene in individuals without atopy, such that hybridisation only occurs when the said variant gene is present in the sample.
- 15 8. A method according to claim 2 wherein the analysis of the nucleic acid sample for the presence or absence of said variant gene is carried out by direct sequencing of the gene sequence which encodes the E237G polymorphism, and wherein the nucleotide sequences for 5' to 3' and 3' to 5' sequencing are amplified prior to said direct sequencing.
- 20 9. A method according to claim 2 wherein the analysis of the nucleic acid sample for the presence or absence of said variant gene is carried out by single stranded conformational polymorphism (SSCP) analysis.
- 25 10. A method according to claim 1 wherein the presence or absence of the unusual polymorphic form of the amino acid sequence for FcεRIβ is established by use of a substance which comprises an antibody binding domain which is specific for the unusual polymorphic form.
- 30 11. A method according to claim 10 wherein the substance comprises an antibody.

12. A method according to claim 11 wherein the antibody is either monoclonal or polyclonal.

5 13. An oligonucleotide hybridization probe for diagnosing an individual as having atopy or a predisposition thereto, which probe comprises a sequence of nucleotides such that under suitably stringent conditions the probe: (i) specifically binds to an unusual variant form of the gene which codes for FcεRIβ the variant form comprising a variation in exon 7 which encodes a glycine at amino acid residue 237 instead of glutamic acid which appears at residue 237 in individuals  
10 without atopy; and (ii) fails to bind substantially to the genes which code for FcεRIβ and derive from individuals without atopy.

14. A pair of oligonucleotide primers for amplification of an unusual variant form of the gene which codes for FcεRIβ, for diagnosing an individual as having  
15 atopy or a predisposition thereto, wherein the variant form comprises a variation in exon 7 which encodes a glycine at amino acid residue 237 instead of glutamic acid which appears at residue 237 in individuals without atopy; wherein the first member of the primer pair comprises a nucleotide sequence which hybridises under suitably stringent conditions to a substantially complementary  
20 nucleotide sequence which is proximal to, and 5' of the codons for amino acid residue 237; and the second member of the primer pair comprises a nucleotide sequence which hybridises under suitably stringent conditions to a substantially complementary nucleotide sequence which is proximal to, and 3' of the codon for amino acid  
25 residue 237.

15. A pair of oligonucleotide primers for amplification of an unusual variant form of the gene which codes for FcεRIβ, for diagnosing an individual as having atopy or a predisposition thereto, wherein the variant form comprises a variation  
30 in exon 7 which encodes a glycine at amino acid 237 instead of glutamic acid which appears at residue 237 in individuals without atopy; wherein a member of the primer pair comprises a nucleotide sequence which itself hybridises under

suitable stringent conditions to the unusual sequence of nucleotides which give rise to said variant gene such that an amplification reaction employing such a member of a primer pair will only proceed where said variant gene is present in a sample.

5      16. A pair of primers according to claim 15 which in an amplification system function to amplify said unusual variant form, but fail to substantially amplify an equivalent gene which codes for FcεRIβ and derives from individuals without atopy.

10      17. An assay kit which comprises an oligonucleotide hybridisation probe according to claim 13.

18. An assay kit which comprises a pair of oligonucleotide primers according to any one of claims 14 to 16.

15      19. An assay kit which comprises an oligonucleotide hybridisation probe according to claim 13 along with a pair of oligonucleotide primers according to any one of claims 14 to 16.

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 96/02095

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12Q1/68 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,95 05481 (ISSIS INNOVATION LTD.) 23 February 1995 cited in the application see the whole document ---	1-19
A	EP,A,0 649 910 (SUMITOMO ELECTRIC INDUSTRIES LTD.) 26 April 1995 ---	1-19
T	HUMAN MOLECULAR GENETICS, vol. 5, no. 7, July 1996, pages 939-62, XP000616300 HILL R ET AL: "A new variant of the B subunit of the high-affinity receptor for immunoglobulin E(FcεRI-beta E237G): associations with measures of atopy and bronchial hyper-responsiveness " see the whole document --- -/--	1-19

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \*&\* document member of the same patent family

Date of the actual completion of the international search

29 January 1997

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 96/02095

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROC. NATL ACAD SCI USA,  vol. 89, no. 8, April 1989,  pages 2766-70, XP000310584  ORITA M ET AL: "Detection of  polymorphisms of human DNA by gel  electrophoresis as single-strand  conformation polymorphisms"  see the whole document</p> <p style="text-align: center;">-----</p>	9



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 96/02095

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9505481	23-02-95	NONE	
EP-A-649910	26-04-95	JP-A- 7194400	01-08-95